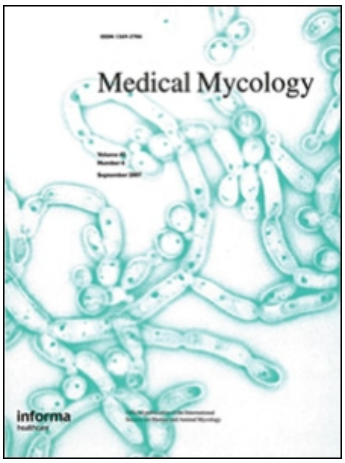

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Using aCGH to study intraspecific genetic variability in two pathogenic molds, *Aspergillus fumigatus* and *Aspergillus flavus*

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We have examined the feasibility of using array comparative genomic hybridization (aCGH) to explore intraspecific genetic variability at the genomic level in two pathogenic molds, *Aspergillus fumigatus* and *Aspergillus flavus*. Our analysis showed that strain-specific genes may comprise up to 2% of their genomes in comparison to isolates from different vegetative (heterokaryon) compatibility groups (VCGs). In contrast, isolates with the same VCG affiliations have almost identical gene content. Most isolate-specific genes are annotated as 'hypothetical' and located in a few large subtelomeric indels. The list includes highly polymorphic loci that contain putative *het* (heterokaryon compatibility) loci, which determine the individual's VCG during parasexual crossing. Incidentally, VCGs in both species seem to be significantly associated with either alpha or HMG mating type (Chi-square test, $P = 0.05$). In conclusion CGH can be used to effectively to identify isolate-specific genes in *Aspergillus* species. Preliminary evidence suggests that gene flow in both species is largely constrained by VCG boundaries, although further VCG sampling is required to confirm this observation.

Keywords *Aspergillus*, array CGH, heterokaryon incompatibility, mating type, PCD

Introduction

Aspergillus fumigatus is the most common invasive mold infection in immunocompromised individuals [1]. After *A. fumigatus*, *Aspergillus flavus* is the second leading cause of both invasive and non-invasive aspergillosis [2]. It can also attack wounded plants and is the major producer of aflatoxin, which has linked to many human and animal diseases [3]. The two species have been also associated with asthma and sinusitis events in allergic individuals. *A. fumigatus* and *A. flavus* populations are highly polymorphic in nature despite their apparent asexuality, which may have clinical significance in terms of mode of infection, drug resistance or virulence. Phenotypic characterization showed that both *A. fumigatus* and *A. flavus* isolates vary in their pathogenicity and resistance to antifungal drugs [2,4]. *A. flavus isolates* form two genetically distinct groups, I and II. *Aspergillus oryzae* strains appear to be part of a monophyletic *A. flavus* group I and can be distinguished by subtle morphological differences [5,6]. Taken together these observations suggest that genomes of *A. fumigatus* and *A. flavus* isolates contain differential genetic traits such as strain-specific genes responsible for these differences.

The observed intraspecific variability in asexual species such as *A. fumigatus* and *A. flavus* may be attributed to restricted gene flow within populations,

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which appears to be limited to isolates in the same vegetative compatibility group (VCG) [7]. Hyphal fusion during parasexual crossing between individuals from different VCGs is prevented by triggering an apoptosis-like reaction called heterokaryon (vegetative) incompatibility. The fate of the heterokaryotic cell is determined by highly polymorphic heterokaryon incompatibility (*het*) loci. Isolates from the same VCG carry identical *het* alleles, while isolates from different VCGs carry different *het* alleles. *Aspergillus* species appear to have a unique set of putative *het* loci [8–10], which are different from the *het* loci identified in two other filamentous ascomycetes, *Neurospora crassa* and *Penicillium anserina* [11,12]. The extent of genetic variability among *A. fumigatus* and *A. flavus* VCGs and their clinical significance is unknown.

Until recently, genetic diversity among *Aspergillus* species and strains was studied using a small number of variable loci with techniques such as randomly amplified polymorphic DNA typing (RAPD), sequence-specific DNA primer (SSDP), polymorphic microsatellite markers (PMM), restriction fragment length polymorphism (RFLP) and sequencing of intergenic loci [13,14]. The availability the genomic sequences of two *A. fumigatus* [4,8,15] and two *A. flavus, A.oryzae* isolates [16,17] allowed us to examine genetic diversity within these species at the genomic level [18]. Genome sequencing however is an expensive and laborious technology. To circumvent the need to sequence multiple strains, a more cost-effective method called array-based comparative genomic hybridization (aCGH) was recently introduced. It was first applied to identify variation in gene content in uncharacterized bacterial strains by comparison with a reference genome [19]. It has been also used successfully for a number of fungal species such as *Candida*, *Saccharomyces* and *Cryptococcus* species [20–24]. Here, we used the aCGH technology determine gene content among four *A. fumigatus* and two *A. flavus* isolates and to explore putative associations between VCGs, *het* loci and mating types.

Materials and methods

Strains and growth conditions

In this study we used *A. fumigatus* strain A1163, a derivative (uracil auxotroph) of a clinical isolate CEA10 (CBS144.89) [25,26] and three *A. fumigatus* wild-type clinical isolates: Af293, Af294 (NCPF 7102) and Af71 (NCPF 7098). *A. fumigatus* Af293 and *A. flavus* (NRRL3357) were used as aCGH index strains. Other *A. flavus* strains used in this study are listed in Table 1. All strains were grown in triplicate at 25°C in liquid minimal media as described previously [27].

Genomic DNA isolation and aCGH

Genomic DNA (gDNA) from target genomes was purified using the DNeasy Tissue kit (Qiagen), labeled with Cy3-dye, and together with the Cy5-labeled reference strain genome, are hybridized to a single DNA microarray (with a dye-flip replicate) as previously described [15]. The Log2 ratios of hybridization signals between target and reference genomes have been shown to correlate with sequence identity and with the presence/absence of genes in the target strain. The results are being submitted to the Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo).

Whole genome oligo microarrays

The design of the whole genome PCR amplicon coding sequence (CDS) 70-mer oligo microarray for *A. fumigatus* Af293 was described previously in [15]. Similarly, the *A. flavus* 70-mer oligo microarray was designed based on CDS annotated from the *A. flavus* NRRL 3357 whole genome sequence (36.5 Mb) [17]. At least one oligo was designed from each gene model larger then 100 amino acids. For genes with more than one exon, oligos were designed based on the second exon sequence. For genes bigger than 4 kb, two oligos were designed from 5′ and 3′ ends. A total of 12,160 oligos were synthesized by Invitrogen. They represent 12,125 sequences, including 11,837 *A. flavus* genes, 278 *A. oryzae*–specific genes (not present in *A. flavus*), and 10 corn genes that show resistance to *A. flavus*. The oligos were printed in duplicate on UltraGAPs amino-silane coated slides using a high density automated arrayer (Intelligent Automation, Cambridge, MA 02139).

In silico *analysis*

Unique, duplicated and divergent genes in Af293 were identified as described in [8]. Briefly Af293 CDSs were aligned against A1163 assemblies using a genomic mapping and alignment program (GMAP) as implemented in PASA [28] with default parameters Expression values from aCGH experiments were analyzed by the MIDAS module within TM4 (http://TM4.org) [29]. Genes with log 2 expression ratios above 2 were considered as absent in the reference isolate.

To identify Single Nucleotide Polymorphisms (SNPs), all CDS, introns and intergenic regions from Af293 and A1163 were clustered into three respective groups [30]. The DNA types were further filtered using a similarity cutoff of 80% of bases between the two

Table 1 *Aspergillus flavus* strains used in this study and mating type genes

VCG name	Isolate name	Area	State	Year	Mating type
DV06	Tester 2 (cnx)	Danevang	TX, USA	1999	HMG
DV06	Danevang B-E	Danevang	TX, USA	1999	HMG
DV06	30401 Sec9-1	Goodyear	AZ, USA	2002	HMG
BS07	Tester1 (niaD)	Bayside	TX, USA	1999	HMG
BS07	Gulfcoast B-B	Gulfcoast	TX, USA	1999	HMG
BS07	Brownsville 2-O	Riogrande Valley	TX, USA	1999	HMG
B	Tester 1 (cnx)	N. Yuma Valley	AZ, USA	1990	alpha
B	32103-2406-A-G	S. Yuma Valley	AZ, USA	2002	alpha
B	Bernard-1-J	Upper Coast	TX, USA	2001	alpha
n/a	NRRL 3357	n/a	GA, USA	n/a	alpha
n/a	A. oryzae	n/a	Japan	n/a	alpha

genomes of the same type. If the features differed less than 10% in overall length the features were considered clustered and were used for calculation of SNPs. Features that differed more than 10% were analyzed manually. All clusters of each feature type were aligned using ClustalW and SNPs were assigned where the aligned sequences had a change in a nucleotide at a specific location in the alignment.

PCR mating type survey

gDNA from ten *A. flavus* isolates and *A. oryzae* was used in separate polymerase chain reactions (PCR) with four sets of primers designed based the *A. flavus* NRRL 3357 sequence (Table 3). Reaction samples (25 µl) contained 0.25 µM (2 ng) each primer and 200 ng genomic DNA. PCR reactions with MAT-alpha specific primers (sets 1 and 2) were conducted with SuperMix High Fidelity (Invitrogen, 22 U/ml DNA polymerase in 66 mM Tris-SO_4 (pH 9.1 at 25°C), 19.8 mM $(NH_4)_2SO_4$, 2.2 mM $MgSO_4$, 220 µM of each dNTP to a final volume of 25 µl. The reaction had the cycling parameters of 2 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C with reaction finishing with 10 min at 72°C.

The PCR reactions that targeted gene regions flanking the MAT locus (sets 3 and 4) were conducted in 1 × Herculase® reaction buffer (Stratagene) with 2.5U Herculase® enhanced DNA polymerase, 0.5% DMSO, 200 µM of each dNTP to a final volume of 25 µl. Cycling parameters were 2 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, 10 min at 72°C with reaction finishing with 10 min at 72°C. Samples from PCR products were digested with restriction endonuclease SpeI.

A. fumigatus strains were surveyed using MAT-specific primers AFM1-3, AFM31, AFM32, AF51 and AF52 as described in [31]. All PCR and restriction digestion products were visualized by gel electrophoresis on either 3% NuSieve® 3:1 Agarose or 1% Agarose stained with ethidum bromide.

Results and discussion

A. fumigatus *isolate-specific genes identified by aCGH*

Access to the two recently sequenced strains of *A. fumigatus* (Af293 and A1163) [4,8,15] gave us an opportunity to evaluate the feasibility of using aCGH to explore differences in gene content among fungal isolates. We used the Log2 ratios of hybridization signals between target (Af293) and reference (A1163) genomes as a measure of sequence identity. Genes that showed negative ratios less than −2 were considered

Table 2 *Aspergillus fumigatus* strains used in this study and mating type genes

VCG name	Isolate name	Source	State/Country	Year	Mating type
FU06	WSA-1195	Clinical	CT, USA	n/a	HMG
FU06	WSA-270	Clinical, lung	India	n/a	HMG
FU06	WSA-449	Clinical	CA, USA	n/a	HMG
FU06	WSA-172	Clinical	USA	n/a	HMG
FU04	Af293	Clinical, lung	Shrewsbury, UK	1993	HMG
FU01	A1163	Clinical*	France	1989	alpha
n/a	Af294	Clinical, lung	Paris, France	1994	alpha
n/a	Af71	Clinical, lung	CA, USA	1989	HMG

*A1163 is a uracil auxotroph of a clinical isolate CEA10 (CBS144.89) converted to *pyrG*+ via the ectopic insertion of the *A. niger pyrG* gene.

Table 3 Primers used in the PCR mating-type survey in *Aspergillus flavus* strains

Primer set-target	ID	Sequence (5′ to 3′)	Product size (Kb)
Set 1-internal alpha	100	ATTTCGACCAACTATTCTGTCAATG	0.5
	102	CACTGGAAGCCTATGACTTCTCTAG	
Set 2-internal alpha	101	ATAGTGATGTTTCTCTGGAACCTTTTCT	0.5
	103	CAACCATACTATTGTAACCGATGTTTAC	
Set 3-external	105	GTTTTGAGGAAGCTTAGGAATAGTGATA	7.5
	106	TTAAGCCTAAAGGTGTTGTTAGTAGTCA	
Set 4-external	107	ATCATATCGGCTAGCTCTAGGTTCT	8
	108	ATCATTCAGTCTTTCCTGGATATTTACT	

absent in the target strain. Using this cut-off, we identified 109 Af293-specific genes (Table 4). This is less than 143 Af293-specific genes predicted by the PASA-based comparative genomic analysis [8]. In the aCGH analysis, 12 genes appeared to be false positives (Af293-specific genes that hybridized with the A1163 gDNA) and 20 genes seem to be false negatives (Af293-specific genes that hybridized with the A1163 gDNA). The discrepancies can be explained by the presence of divergent genes, annotation and sequencing errors, limitations of the current aCGH technology and our implementation. It should be noted however that only 117 of those were present on the array. Taken together these results suggest at least a 90% overlap between the two Af293-specific gene sets and that aCGH is a reliable method for detecting isolate-specific genes.

Similar results were obtained for the two other *A. fumigatus* isolates, Af71 and Af294 (Table 4). In all three aCGH datasets, most Af293-specific genes tend to be located within 300 Kb from chromosome ends (Table 4). They are often found clustered in a few subtelomeric indels including a large region on the left arm of chromosome 1, which seem to be the most variable segment of the *A. fumigatus* Af293 genome. About one third of Af293-specific genes are differentially expressed under various conditions (W. Nierman and G. May, unpublished), which is lower than the genome average (44%), suggesting that some of them may be functional. In contrast, 55% of duplicated genes are differentially expressed, which is lower than genome average.

Further analysis confirmed significant variability of the *A. fumigatus* gene content and showed that the number of Af293-specific genes depends on the number of target genomes. It revealed 63 Af293-specific genes (0.7% of the genome), which appear absent in all three target genomes (Af71, Af294 and A1163). Most of these genes [33] are annotated as hypothetical or conserved hypothetical, although some appear to function in secondary metabolism. The genes involved in secondary metabolism are found together in a putative secondary metabolism cluster (AFUA_3G02550 – AFUA_3G02650) located in the middle of chromosome 3. Interestingly, the list also included three putative *het* genes from the highly polymorphic *rosA* locus, suggesting that the isolated belong to different VCG groups as discussed below.

A. flavus *isolate-specific genes identified by aCGH*

Previous comparative genomic analysis demonstrated that the genome of *A. flavus* NRRL 3357 contain hundreds of unique genes with respect to *A. oryzae* [18]. To evaluate the extent of genetic variability within the same VCG, we performed aCGH with two *A. flavus* strains from the same VCG (DV06), but isolated two years apart and from different regions in the United

Table 4 Numbers of *Aspergillus fumigatus* Af293 genes not detected in other isolates

Method of identification	No. strain-specific genes	No. expressed genes	Expressed genes (%)	No. subtelomeric genes	Subtelomeric genes (%)
A1163 (PASA)	143 (117*)	51	36	87	61
A1163 duplicated	29	16	55	13	45
A1163 divergent	42	16	38	18	43
A1163 (CGH)	109 (98**)	51	47	57	52
Af294 (CGH)	132	58	44	69	52
Af71 (CGH)	192	82	43	121	63
Total	9632	4246	44	1509	16

*Numbers of genes with a gene probe on the Af293 array.
**Number of genes that were identified as Af293-specific by the PASA analysis.

States (Texas and Arizona). gDNA from *A. flavus* strains Danevang B-E and 30401 Sec9-1 was hybridized with the *A. flavus* NRRL 3357 based array. Expression ratios were estimated based on hybridization signals for Danevang B-E relative to 30401 Sec9-1 and vice versa.

aCGH analysis of these two DV06 strains demonstrate that their gene content is almost identical. We have detected only two genes (AFLA_005140 and AFLA_030730) with log2 ratios less than −2, which were presumably highly divergent or absent in Danevang B-E. This is consistent with the view that genetic exchange in asexual filamentous fungi is thought to be limited by VCG affiliations. It was shown previously that *A. flavus* isolates from the same VCG have similar sets of single nucleotide polymorphisms regardless of isolate origin [7]. Both the current and previous observations support the hypothesis that the vegetative incompatibility system in *A. flavus* genetically isolates VCGs, and that members of the same VCG are highly similar at the genetic level.

New candidate het genes in A. fumigatus

Despite the absence of meiosis during the life cycle of asexual fungi, recombination and genetic variation can occur by a mechanism called parasexuality. Different individuals are capable of undergoing hyphal fusion with each other to form a vegetative heterokaryon, where genetically different nuclei coexist in a common cytoplasm. Parasexual crossing between genetically distant individuals is prevented in some filamentous ascomycetes by triggering a programmed cell death (PCD) reaction called heterokaryon incompatibility. The reaction is characterized by severe growth inhibition, repression of asexual sporulation, hyphal compartmentation and apoptotic-type death in the heterokaryotic cell. It is thought to act as a self/non-self recognition system that prevents hyphal fusion and genetic exchange between strains that carry different alleles at vegetative (heterokaryon) incompatibility (*het*) loci [11,12].

The Aspergilli heterokaryon incompatibility system is thought to be similar to the one described in *N. crassa* and *P. anserina* [9,32,33]. Previous comparative genomic analyses however demonstrated that *Aspergillus* orthologs of known *N. crassa* and *P. anserina het* genes [9,32] do not show allelic polymorphism. This suggests that they do not act as a *bona fide het* genes in *A. fumigatus* and in other aspergilli. Nonetheless, Aspergilli have been shown to undergo heterokaryon incompatibility. Thus *A. nidulans* has at least eight *het* loci, which have not been characterized at the molecular level [33].

We previously used comparative genomic analysis of Af293 and 1163 to identify three *A. fumigatus* putative *het* loci (AFUA_2G00910, AFUA_2G17420, AFUA_6G07000-30), which contained genes with low sequence identity between Af293 and A1163 orthologs [4]. Manual examination of their nucleotide alignments confirmed that these loci exhibit patterns of allelic polymorphism similar to those observed in *N. crassa* and *P. anserina het* loci (e.g., *N. crassa* HET-C [34]). Incidentally all three *A. fumigatus* loci encoded STAND (found in NATCH and NB-ARC NTPases) [35]) and/or Pfs (found in nucleosidases and phosphoribosyltransferases) [36] domains, which were predicted to function in heterokaryon incompatibility [9]. They also exhibited patterns of trans-specific polymorphisms found in known *het* genes from other species (e.g., [37]). For example, one putative *het* locus (*rosA*) also appears to be polymorphic in *A. oryzae* and *A. flavus.*

To identify additional candidate *het* genes in *A. fumigatus*, we have examined genes with varying aCGH hybridization patterns with A1163, Af294 or Af71 gDNA. In total we identified eight such loci (Table 5) including two that showed low sequence identity between Af293 and A1163 alleles (AFUA_6G11710 and AFUA_6G13820). Further characterization of these candidate *Aspergillus*-specific *het* genes will facilitate our understanding of the initial stages of PCD and of genetic barriers to mating and parasexual crossing in this genus and thus facilitate genetic manipulations. It may help expand the range of currently available treatments for invasive aspergillosis, since several antifungal agents including amphotericin B [38] and rapamycin [39] have already been shown to induce cell death reactions similar to vegetative incompatibility. In addition, better understanding of this reaction may provide further insights into evolution of PCD in the eukaryotic cell.

Association between VCGs and mating type (MAT) genes

In addition to parasexual crossing, some *Aspergillus* species can undergo sexual reproduction or mating. Unlike parasexual hyphal fusions, mating involves fruiting bodies called cleistothecia that contain ascospores. In heterothallic (obligate sexual outbreeding) fungi, only individuals with different mating types can engage in sexual reproduction. Heterothallic fungal populations are divided into different mating types (typically alpha or HMG) encoded by their MAT loci, which determine mating self-incompatibility. Although *A. fumigatus* and *A. flavus* species have been considered asexual organisms, their MAT loci have an idiomorph structure characteristic of heterothallic fungi

Table 5 *Aspergillus fumigatus* Af293 genes with mosaic aCGH patterns among A1163, A71 and A294 isolates

Af293 accession	Protein description	aCGH data (log2 ratios)			Comparison to A1163 alleles	
		Af294	Af71	A1163	Identity (%)	A1163 accession
AFUA_2G00910	Pfs-NB-ARC-TPR domain protein	0.03	0.25	0.04	85.65	AFUB_017990
AFUA_2G17420	Pfs-NB-ARC domain protein	−0.1	−4.57	−3.06	45.33	AFUB_033090
AFUA_6G07000	Conserved hypothetical protein	n/a	n/a	n/a	38.89	AFUB_072925
AFUA_6G07010	Transcription factor RosA	−3.68	−3.63	−1.69	54.89	AFUB_072930
AFUA_6G07020	SAM and PH domain protein	−2.97	−3.23	−2.21	55.86	AFUB_072940
AFUA_6G07030	NACHT-Ankyrin domain protein	−3.06	−3.15	−2.3	54.82	AFUB_072950
AFUA_6G11710	Conserved hypothetical protein	−0.3	−0.26	−1.28	90.67	AFUB_077710
AFUA_6G13820	Conserved hypothetical protein	−2.18	−2.13	−1.71	32.1	AFUB_000910
AFUA_5G13760	Hypothetical protein	0.07	−1.53	0.15	91.46	AFUB_061500
AFUA_4G01110	1,3-beta glucanase	−0.4	−4.36	−0.89	94.03	AFUB_101620
AFUA_3G15190	Nitrate reductase	−0.14	−3.52	−0.05	97.91	AFUB_034030
AFUA_3G15200	Conserved hypothetical protein	0.13	−3.71	−0.07	100	AFUB_034020
AFUA_4G01120	Hypothetical protein	0.09	−3.08	0.17	100	AFUB_101630
AFUA_8G01500	Pfs domain protein	−2.22	−2.33	−0.06	100	AFUB_085110

[15,16,31,40,41]. Furthermore, analysis of 290 *A. fumigatus* isolates demonstrated the presence of alpha and HMG genotypes in clinical and environmental isolates in similar proportions [31]. These and several other studies have led to conclusion that both *A. fumigatus* and *A. oryzae* may be capable of sexual reproduction.

To find out if *A. flavus* VCG affiliations may correlate with alpha or HMG mating types, we performed a mating-type PCR survey of nine *A. flavus* isolates from three VCG groups using internal and external sets of primers designed with the *A. flavus* NRRL 3357 MAT region and its flanking sequences. The survey has demonstrated that all isolates from VCG B have the HMG allele, which is also found in the sequenced isolates of *A. flavus* NRRL 3357 and *A. oryzae* RIB40 (Table 1). In contrast, all VCGs DV6 and BS07 strains have the alpha allele. Statistical analysis shows these VCGs are significantly associated with certain mating types (Chi-square test, $P = 0.05$). We calculated Chi-square assuming there are only two VCGs, because as DV06 and BS07 are related.

Similar results were obtained for *A. fumigatus* isolates WSA-1195, WSA-270 and WSA-449 (Table 2). These strains were shown to belong to the same VCG (P. Cotty, personal communication), but isolated from different states or countries. All three show the same size band on the agarose gel with HMG-specific primers. Incidentally, the same isolates were shown previously to have the *het* alleles at AFUA_2G00910, AFUA_2G17420, AFUA_6G0700- AFUA_6G0730 loci [4].

The highly unequal distribution of mating types in our PCR survey agrees with a clonal nature of each VCG examined here. These patterns also imply that *A. flavus* and *A. fumigatus* may rarely recombine sexually. Independent segregation of MAT and *het* alleles in natural populations is one of the key characteristics of heterothallic (obligate sexual outbreeding) fungi as has been shown for *Neurospora tetrasperma* and *N. crassa* [42,43].

Conclusions

This study demonstrates that aCGH approaches are effective in uncovering intraspecific differences in gene content, which can facilitate discovery of new polymorphic markers and identification of new virulence factors. Unexpectedly, these approaches can also provide significant insights into sexual and vegetative compatibility systems, which regulate gene flow by providing genetic barriers to prevent selfing,and parasexual crossing between individuals. Our results suggest that the vegetative incompatibility system may significantly limit genetic exchange between dissimilar strains of *A. fumigatus* and *A. flavus* and that these two species rarely, if ever, recombine sexually. These observations may have important implications for biological control strategies such as seeding fields with large quantities of natural nontoxigenic *A. flavus* strains. Future research involving more extensive VCG sampling and different *Aspergillus* species is required to confirm these preliminary observations.

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